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(57) Abstract

A high-throughput method for screening compounds for antiviral activity involves cultivating cells capable of expressing viral genes in cell culture in microtiter plate wells in the presence of test compounds and detecting the level of viral gene expression by a quantitative nucleic acid amplification technique.

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HIGH-THROUGHPUT METHOD FOR SCREENING FOR ANTIVIRAL ACTIVITY

This application is based on provisional application serial number 60/080,894, filed April 7, 1998.

Background of the Invention

The present invention relates to a high-throughput method for screening potentially active compounds for antiviral activity. More particularly, the invention relates to an *in vitro* antiviral screening method employing virus-producing cell lines.

The antiviral activities of a number of drugs, including many of the drugs currently used for treating HIV disease, have been discovered by screening procedures using cell cultures infected with the virus. Various formats and end points of detection have been used in such screening methods. For example, activity against cytotoxic viruses may be assessed by determining the ability of a compound to inhibit virus-induced cell death. Other methods measure the effect of a putative antiviral drug on the rate of production virus particles by infected cells. Alternatively, the effect of compounds on expression of viral genes may be used as an indication of antiviral activity.

Although structure-function relationships and rational drug design have been successfully employed in antiviral research, a major component of such research still involves high-volume screening of large numbers

of compounds. Accordingly, antiviral screening methods should be robust, sensitive, fast and capable of accommodating large numbers of samples simultaneously. In addition, experimental drugs often are available only in small quantities. A successful screening method therefore advantageously uses small cell culture volumes and sensitive detection methods. Currently, the standard 96-well microtiter plate format is preferred for these types of procedures.

A necessary component of such antiviral screening methods is a cell culture that can be cultivated efficiently in vitro and which can be infected with either the virus of interest or a related virus that simulates the in vivo viral infection for which a treatment is sought. Viral replication in the infected cell line should mimic that which occurs in vivo. For many viruses, chronically infected cell lines, i.e., those in which the viral genome is stably introduced into the cells, are preferred. Such cell lines should either produce virus continuously or viral production should be inducible, so that antiviral activity can be determined at one or more stages of the viral replication cycle.

A virus of particular interest for antiviral screening is the Hepatitis-B virus (HBV). HBV infection is widespread, with over two million individuals chronically infected. HBV infection causes both acute and chronic liver disease and has been linked to liver cancer. Current therapies include treatment with interferons and certain nucleoside analogs; however, neither of these treatments is

entirely effective. Accordingly, a substantial need exists for new and more effective anti-HBV drugs.

In vitro cell culture screening methods for HBV have been described in the scientific literature. Sells et al., Proc. Natl. Acad. Sci. USA, 84:1005-1009 (1987), starting with a hepatoma cell line designated HepG2 (American Type Culture Collection Access No. ATCC HB 8065), have developed a producer cell line, designated HepG2-2.2.15, containing the HBV genome. Screening methods using the HepG2-2.2.15 cell line have been reported by Korba, B. et al., Antiviral Research, 15: 217-238(1991) and Korba, B., et al., Antiviral Research, 19:55-70(1992). The procedures described in these publications involve cultivating the infected cells in the presence of the compound being tested. The cells were grown to confluency and the putative antiviral compound was added in ten consecutive daily doses. Samples of culture medium were taken at day zero (before drug addition) and after three, six, and ten days of treatment. The samples were analyzed by a slot-blot hybridization technique for extra-cellular (virion) HBV DNA. Results were reported as the concentration which induced a ninety percent decrease in the level of HBV DNA (EC_{90}).

While the procedures described in these publications represent an advance over prior HBV screening methods, they still suffer from certain disadvantages. Most significantly, the relative insensitivity of the hybridization technique used for detecting viral DNA requires that relatively high culture volumes and cell densities be employed. The references describe the use of a 24-well culture plate

in which individual wells have volumes of about 500 µl. These relatively large culture volumes and cell densities, in turn, require relatively large quantities of the putative antiviral compound being tested. Accordingly, a need exists for a high-throughput, low volume rapid and reliable in vitro screening method for HBV and other viruses.

Summary of the Invention

In accordance with the present invention, a method for simultaneously screening a plurality of compounds for antiviral activity against a virus involves the steps of:

(1) cultivating cells, which are capable of expressing viral genes, in culture medium in wells of a microtiter plate, each well containing a culture volume of from about 50 µl to about 250 µl;

(2) adding each compound being tested to a separate cell- containing well at a concentration at which the antiviral activity is to be tested;

(3) culturing the cells for a time sufficient for the cells to express viral genes;

(4) subjecting a sample of cell-free medium of each well to quantitative nucleic acid amplification of a sequence from at least one viral gene to determine the extent of expression for the viral gene in each well; and

(5) correlating the extent of viral gene expression, as determined by quantitative nucleic acid amplification, to the

concentration of each compound to determine the effectiveness of the compounds in inhibiting viral replication.

It has been found that quantitative nucleic acid amplification provides a sensitive and reliable method for determining the extent of expression of viral gene expression. The sensitivity of this detection method permit the use of very small culture volumes, in contrast to the relatively large culture volumes and over-growth conditions described in the prior art. The small culture volumes, in turn, permit the screening assay to be performed in a microtiter plate format and permit the use of very small amounts of test compounds. By using the microtiter plate format, numerous compounds can be tested simultaneously to provide a high-throughput screening method.

Description of the Drawings

Figure 1 is a representative microtiter plate layout for testing compounds in accordance with the method of the present invention.

Figure 2 is a microtiter plate layout used for determining a drug dose response curve in accordance with a method according to the present invention.

Detailed Description of the Invention

It will be appreciated by those skilled in the art that the screening method described herein may be applied to a wide variety of viruses. Any virus for which an infected or producer cell line can be cultivated in vitro and which expresses viral genes can be employed in this method. Advantageously, the cells employed in the screening method express at least those viral genes that are associated with the phase of viral

replication that is a target for therapeutic interference. Preferably, viral gene expression, packaging and release of viral particles into the medium in which the cells are cultured simulates that which occurs in infected cells in vivo in the natural host. Activity against both DNA and RNA viruses can be determined by the present screening methods. Such viruses include, for example, hepadnaviruses, members of the herpesvirus family, retroviruses, rhabdoviruses, bunyaviruses, flaviviruses, togaviruses, baculoviruses, and the like. The screening method of this invention will be further illustrated and described in detail by reference to a method for screening for activity against the hepadnavirus, HBV.

The HepG2-2.2.15 cell line described by Sells et al. may advantageously be utilized in the present method for screening compounds for activity against HBV. Alternatively, a cell line transformed with the HBV genome under control of an inducible promoter may be used. In the latter system, cells may be grown to a desired cell density and then expression of the HBV genes may be induced by adding a compound that induces expression or by replacing media containing a compound that represses expression with repressor-free media. A cell line containing the HBV genome under control of an inducible promoter is described by Ladner et al., Antimicrobial Agents and Chemotherapy, August 1997, 1715-1720.

The cells may be cultured by techniques well known to those skilled in the art. The cells are cultivated in a nutrient medium that supports cell viability and growth. While the medium may vary, depending upon the

particular cells employed. Suitable media include Dulbecco's modified Eagle's medium ("DMEM") or Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% fetal bovine serum. Culture plates are advantageously maintained in humidified incubation chambers at 37°C in an atmosphere containing 5% carbon dioxide.

Cell-containing media are added to the wells of a microtiter plate. A preferred format for carrying out the screening method of this invention utilizes a conventional 96-well microtiter plate. Each well contains less than about 200 µl, preferably less than about 100 µl of culture medium, e.g., from about 50 µl to about 200 µl, preferably from about 50 µl to about 100 µl of culture medium. When using the 96-well microtiter plate format, it is preferred to use only the internal wells for testing, because of "edge effects" caused by evaporation of media from the outer wells. Accordingly, the outer rows and columns of wells are advantageously filled with media only.

Once plated in the wells, the cells are cultivated to a desired cell density. When using the HepG-2.2.1.5 cells, it is preferred that the cells be grown to a confluent monolayer on the bottom of the plate. Further growth to higher cell densities is usually unnecessary in the present method.

When the cell growth has reached the desired density, the compounds to be tested are added to the microtiter plate wells. It is preferred that each compound being tested be added to a plurality of wells at different concentrations. It is usually desired the compound be added in dilutions that include a useful

dose-response curve. If concentrations are selected appropriately, the results of the method can be expressed as the concentration of the compound which is effective in inhibiting a certain percentage of viral replication, e.g., the EC_{90}. It is also preferred that blank wells, which contain cells and media but do not contain test compound, are included as controls. Positive controls, i.e., compounds which are known to inhibit viral replication, also may be included.

A wide variety of different types of potential antiviral agents may be tested in the method of this invention. For example, chemical agents, such as nucleoside analogs, biological agents such as peptides, proteins or antibodies, natural agents, such as extracts of plants, bacteria and fungi, and many other types of agents may be tested.

In a preferred method, once the desired cell density has been reached, the culture medium is removed from each well and is replaced with culture medium containing the test compound. Cultivation typically spans several days, and it is preferred that culture medium containing test compound be replaced on a periodic, e.g., daily, basis.

The present method permits the use of test compound concentrations in the micromolar range and below. The concentrations generally range from about 1 nanomolar to about 5 millimolar. Concentrations can be adjusted after initial results to obtain a useable dose-response curve.

After the cells have been cultivated in the presence of test compounds for a time sufficient for expression of viral genes, the culture medium of each

well is analyzed by a quantitative nucleic acid application procedure. A nucleic acid sequence that is indicative of replication of the virus is selected for amplification. The nucleic acid sequence may, for example, be a sequence from a gene encoding a core protein, a viral enzyme, such as polymerase, kinase, or protease, an envelope or transmembrane protein, or any other component indicative of viral replication. The nucleic acid amplification technique may be any method that specifically amplifies the nucleic acid of interest, including polymerase chain reaction ("PCR"), ligase chain reaction ("LCR") or nucleic acid specific base amplification ("NASBA"). PCR is the preferred amplification procedure.

The primers used for the amplification are selected so as to amplify a unique segment of a viral gene that is indicative of viral replication. In the case of HBV, it has been found that a segment of the gene encoding the core protein functions particularly well. The amplification primers generally comprise from 8 to about 50, preferably from about 10 to 30 nucleotides. The primers are chosen to amplify a segment containing from about 25 to about 500, preferably from about 50 to about 150 nucleotides. Advantageously, the primers are selected such that the primer template complex has a melting point of about 50°C. Software that assists in primer design is commercially available.

The quantitative nucleic acid amplification technique is preferably a technique which involves monitoring the progress of the nucleic acid amplification by use of an oligonucleotide probe

having a fluorescent reporter molecule and a quencher molecule at either end. The quencher molecule substantially quenches any fluorescence from the reporter molecule when the oligonucleotide probe is a single-stranded form, and the reporter is substantially unquenched whenever the oligonucleotide is a double-stranded form hybridized to the target. This type of probe is sometimes referred to as "TaqMan" probe. Quantitative PCR by this technique is described in U.S. patent 5,538,848 which issued on July 23, 1996 to Livak et al., the disclosure of which is incorporated herein by reference. Related probes and quantitative amplification procedures are described in U.S. patent 5,716,784, which issued on February 10, 1998 to Di Cesare et al. and U.S. patent 5,723,591, which issued on March 3, 1998 to Livak et al., the disclosures of which are incorporated herein by reference. Instruments for carrying out quantitative PCR in microtiter plates are available from PE Applied Biosystems, 850 Lincoln Centre Drive, Foster City, Ca 94404 under the trademark ABI Prism® 7700.

An alternative quantitative nucleic acid amplification procedure is described in U.S. patent 5,219,727, which issued on June 15, 1993 to Wang et al., the disclosure of which is incorporated herein by reference. In this procedure, the amount of a target sequence in a sample is determined by simultaneously amplifying the target sequence and an internal standard nucleic acid segment. The amount of amplified DNA from each segment is determined and compared to a standard curve to determine the amount of the target nucleic

acid segment that was present in the sample prior to amplification.

After determining the level of expression of the viral gene in each microtiter well for a given compound, it is correlated to the concentration of the compound as an indicator of the compound's effectiveness in inhibiting viral replication. If the range of concentrations is appropriate and a sufficient number of different concentrations is tested, an EC_{90} or other measure of effectiveness can be calculated.

The method of this invention is further illustrated by the following example, which is intended to illustrate and not limit the invention.

Example

This example describes the use of the HBV producer cell line, HepG2-2.2.15 described by Sells et al. supra for determining the anti-HBV activity of test compounds.

The cells are plated in 96-well microtiter plates at an initial density of 2.5×10^3 cells/100µl in DMEM medium supplemented with 10% fetal bovine serum. To promote cell adherence, the 96-well plates are pre-coated with collagen prior to cell plating. As shown in Figure 1, only the interior wells are utilized to reduce "edge effects" observed during cell culture phase. The exterior wells are filled with complete medium to help minimize sample evaporation. After incubation at 37°C in a humidified, 5% CO_2 environment, for 16-24 hours, the confluent monolayer of HepG2-2.2.15 cells is washed and the medium is replaced by complete medium containing various concentrations of test compound. See Figure 1 as a representative plate

layout for testing compounds at 6 half-log concentrations. Every three days, the culture medium is replaced with fresh medium containing the appropriate diluted drug. Six days following the initial administration of test compound, the cell culture supernatant is collected and clarified by centrifugation using a Sorvall RT-6000D centrifuge, at 1000 RPM for five minutes. The clarified supernatants are then treated with 0.75 µg/ml Pronase for 30 minutes at 37°C to inactivate proteases and with DNAse for 60 minutes at 37°C to degrade unencapsidated DNA. The supernatants then are heated to 95°C for 30 minutes to inactivate the DNAse. Three microliters of clarified supernatant are then subjected to real-time quantitative PCR using the conditions described below.

Virion-associated HBV DNA present in the cell culture supernatants is amplified by PCR using primers derived from HBV strain AYW. The sequences of the forward and reverse primers are:

Forward primer: 5'-CCA AAT GCC CCT ATC CTA TCA-3'
Reverse primer: 5'-GAG GCG AGG GAG TTC TTC TTC TA-3'

The PCR-amplified HBV DNA is detected in real time (i.e., at each PCR thermocycle step) by monitoring increases in florescence signals that result from an exonucleolytic degradation of a quenched fluorescent probe molecule following hybridization of the probe to the amplified HBV DNA. The probe used is of the TaqMan design and is complementary to DNA sequences present in the HBV DNA region amplified. Its sequence is:

5'-FAM-CG GAA ACT ACT GTT GTT AGA CGA CGA GGC AG-TAMRA-3' where FAM is 6-carboxyfluorescein phosphoramidite and TAMRA is 6-carboxytetramethylrhodamine. The

clarified supernatent (3 microliters) is analyzed directly, without DNA extraction, in a 50 microliter PCR reaction, using standard PCR reagents and conditions. The plate layout used for determining a drug dose response curve is provided in Figure 2. For each PCR amplification, a standard curve is simultaneously generated for several log dilutions of a purified 1.2 kbp HBV ayw subgenomic fragment. The standard curve ranges from $1x10^6$ to $1x10^1$ nominal copy equivalence for PCR reaction.

CLAIMS

What is claimed is:

1. a method for simultaneously screening a plurality of compounds for antiviral activity against a virus, which comprises the steps of:

(a) cultivating cells, which are capable of expressing viral genes, in culture medium in wells of a microtiter plate, each well containing a culture volume from about 50 µl to about 250 µl;

(b) adding each compound being tested to a separate cell- containing well at a concentration at which the antiviral activity is to be tested;

(c) culturing the cells for a time sufficient for the cells to express viral genes;

(d) subjecting a sample of cell-free medium of each well to quantitative nucleic acid amplification of a segment from at least one viral gene to determine the extent of expression for the viral gene in each well; and

(e) correlating the extent of viral gene expression, as determined by quantitative nucleic acid amplification, to the concentration of each compound to determine the effectiveness of the compounds in inhibiting viral replication.

2. The method of claim 1, wherein each microtiter plate well contains less than about 100 µl of culture medium.

3. The method of claim 1, wherein, in step (a) the cells are cultivated until a confluent monolayer of cells is produced on the bottom of the wells of the microtiter plate.

4. The method of claim 1, wherein each compound is tested at a plurality of different concentrations.

5. The method of claim 2, wherein the quantitative nucleic amplification is quantitative polymerase chain reaction.

6. The method of claim 5, wherein the progress of the polymerase chain reaction amplification is monitored by use of an oligonucleotide probe which is complementary to the viral gene segment being amplified and which has a fluorescent reporter molecule and a quencher molecule.

7. The method of claim 1 or 5, wherein the virus is HBV.

8. The method of claim 7, wherein the quantitative nucleic amplification amplifies a segment of the core gene of the HBV genome.

9. The method of claim 7, wherein the cells are from the HepG2-2.2.15 cell line.

10. The method of claim 9, wherein, in step (a) the cells are cultivated until a confluent monolayer of HepG2-2.2.15 cells is produced on the bottom of the wells of the microtiter plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA
B	MEDIA	CELLS + DRUG 1 100 µM	CELLS + DRUG 1 100 µM	CELLS + DRUG 1 100 µM	CELLS + DRUG 2 100 µM	CELLS + DRUG 2 100 µM	CELLS + DRUG 2 100 µM	CELLS + DRUG 3 100 µM	CELLS + DRUG 3 100 µM	CELLS + DRUG 3 100 µM	VC	MEDIA
C	MEDIA	CELLS + DRUG 1 32 µM	CELLS + DRUG 1 32 µM	CELLS + DRUG 1 32 µM	CELLS + DRUG 2 32 µM	CELLS + DRUG 2 32 µM	CELLS + DRUG 2 32 µM	CELLS + DRUG 3 32 µM	CELLS + DRUG 3 32 µM	CELLS + DRUG 3 32 µM	VC	MEDIA
D	MEDIA	CELLS + DRUG 1 10 µM	CELLS + DRUG 1 10 µM	CELLS + DRUG 1 10 µM	CELLS + DRUG 2 10 µM	CELLS + DRUG 2 10 µM	CELLS + DRUG 2 10 µM	CELLS + DRUG 3 10 µM	CELLS + DRUG 3 10 µM	CELLS + DRUG 3 10 µM	VC	MEDIA
E	MEDIA	CELLS + DRUG 1 3.2 µM	CELLS + DRUG 1 3.2 µM	CELLS + DRUG 1 3.2 µM	CELLS + DRUG 2 3.2 µM	CELLS + DRUG 2 3.2 µM	CELLS + DRUG 2 3.2 µM	CELLS + DRUG 3 3.2 µM	CELLS + DRUG 3 3.2 µM	CELLS + DRUG 3 3.2 µM	VC	MEDIA
F	MEDIA	CELLS + DRUG 1 1 µM	CELLS + DRUG 1 1 µM	CELLS + DRUG 1 1 µM	CELLS + DRUG 2 1 µM	CELLS + DRUG 2 1µM	CELLS + DRUG 2 1 µM	CELLS + DRUG 3 1 µM	CELLS + DRUG 3 1 µM	CELLS + DRUG 3 1 µM	VC	MEDIA
G	MEDIA	CELLS + DRUG 1 320 nM	CELLS + DRUG 1 320 nM	CELLS + DRUG 1 320 nM	CELLS + DRUG 2 320 nM	CELLS + DRUG 2 320 nM	CELLS + DRUG 2 320 nM	CELLS + DRUG 3 320 nM	CELLS + DRUG 3 320 nM	CELLS + DRUG 3 320 nM	VC	MEDIA
H	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA

FIG. 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	Std 10^6	Std 10^5	Std 10^4	Std 10^3	Std 10^2	Std 10^1	Std 10^0	NTC	NTC	NTC	BLANK
B	BLANK	CELLS + DRUG 1 100 µM	CELLS + DRUG 1 100 µM	CELLS + DRUG 1 100 µM	CELLS + DRUG 2 100 µM	CELLS + DRUG 2 100 µM	CELLS + DRUG 2 100 µM	CELLS + DRUG 3 100 µM	CELLS + DRUG 3 100 µM	CELLS + DRUG 3 100 µM	CC	BLANK
C	BLANK	CELLS + DRUG 1 32 µM	CELLS + DRUG 1 32 µM	CELLS + DRUG 1 32 µM	CELLS + DRUG 2 32 µM	CELLS + DRUG 2 32 µM	CELLS + DRUG 2 32 µM	CELLS + DRUG 3 32 µM	CELLS + DRUG 3 32 µM	CELLS + DRUG 3 32 µM	CC	BLANK
D	BLANK	CELLS + DRUG 1 10 µM	CELLS + DRUG 1 10 µM	CELLS + DRUG 1 10 µM	CELLS + DRUG 2 10 µM	CELLS + DRUG 2 10 µM	CELLS + DRUG 2 10 µM	CELLS + DRUG 3 10 µM	CELLS + DRUG 3 10 µM	CELLS + DRUG 3 10 µM	CC	BLANK
E	BLANK	CELLS + DRUG 1 3.2 µM	CELLS + DRUG 1 3.2 µM	CELLS + DRUG 1 3.2 µM	CELLS + DRUG 2 3.2 µM	CELLS + DRUG 2 3.2 µM	CELLS + DRUG 2 3.2 µM	CELLS + DRUG 3 3.2 µM	CELLS + DRUG 3 3.2 µM	CELLS + DRUG 3 3.2 µM	VC	BLANK
F	BLANK	CELLS + DRUG 1 1 µM	CELLS + DRUG 1 1 µM	CELLS + DRUG 1 1 µM	CELLS + DRUG 2 1 µM	CELLS + DRUG 2 1µM	CELLS + DRUG 2 1 µM	CELLS + DRUG 3 1 µM	CELLS + DRUG 3 1 µM	CELLS + DRUG 3 1 µM	VC	BLANK
G	BLANK	CELLS + DRUG 1 320 nM	CELLS + DRUG 1 320 nM	CELLS + DRUG 1 320 nM	CELLS + DRUG 2 320 nM	CELLS + DRUG 2 320 nM	CELLS + DRUG 2 320 nM	CELLS + DRUG 3 320 nM	CELLS + DRUG 3 320 nM	CELLS + DRUG 3 320 nM	VC	BLANK
H	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/07551

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HALLIDAY ET AL.: "DEVELOPMENT OF A MODERATE THROUGHPUT ASSAY USING TaqMan PCR TECHNOLOGY TO IDENTIFY INHIBITORS OF HEPATITIS B VIRUS" ANTIVIRAL RESEARCH , vol. 37, no. 3, March 1998 (1998-03), page A66/96 XP002113789 the whole document ---	1-10
Y	MORRIS ET AL.: "RAPID REVERSE TRANSCRIPTION-PCR DETECTION OF HEPATITIS C VIRUS RNA IN SERUM BY USING THE TaqMan FLUOROGENIC DETECTION SYSTEM" J.CLIN.MICROBIOL., vol. 34, no. 12, 1996, pages 2933-2936, XP002113780 the whole document ---	1-10

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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